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13. ABSTRACT ***(Maximum 200 Words)***

We demonstrated that schwannomin interacts with the eukaryotic initiation factor 3 (eIF3) p110 subunit eight and now investigate the role of schwannomin in eIF3 function. eIF3 is required for the 40 S ribosomal subunit bound to the ternary complex (eIF2-GTP-methionine) to interact with the 5' end of the mRNA, and is required for progression of protein translation. Keeping with our Objectives and Statement of Work, we have 1) verified the interaction of schwannomin and p110 by using the non-transcriptionally-based ras-rescue yeast two-hybrid system, 2) produced and validated two high-quality p110 antibodies, 3) determined the cross-reactivity of our anti-p110 antibodies in mouse rat and human cells, 4) co-immunoprecipitated schwannomin and p110 using one of the new p110 anibodies, 5) cloned the full-length p110 cDNA and validated its expression, 6) co-localized p110 and schwannomin in STS26T schwannoma cells by confocal microscopy, 7) developed a NF2 inducible tet-off MEF cell line for the study on p110 function, 8) narrowed the region of p110 interaction in schwannomin to p110 amino acids 327-635 by yeast two-hybrid, 9) cloned eight plasmids for further narrowing of the p110 region of interaction with schwannomin by in vitro methods, and 10) showed the effects of NF2 mutations on p110 interaction by the yeast two-hybrid method.

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Introduction

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder that predisposes individuals to the development of multiple tumors of the central and peripheral nervous system (Trofatter et al., 1993; Rouleau et al., 1993). Germline mutations in the Nf2 gene are one of the most common causes of inherited tumors of Schwann cell, meningeal or ependymal origin. Nf2 is relatively rare with an incidence of 1 in 40,000 (Evans et al., 1992), but virtually all sporadic schwannomas and most ependymomas and meningiomas harbor Nf2 mutations (Rubio et al., 1994, Ruttledge et al., 1994; Sainz et al., 1994; Bijlsma et al., 1994; Papi et al., 1995; Lekanne-Deprez et al., 1994).

The Nf2 cDNA sequence predicts a protein of 595 amino acids with an estimated molecular weight of 65 kDa, and is spliced in a variety of isoforms with two (isoforms I and II) common in normal cells. The Nf2 protein, called schwannomin or merlin, belongs to a superfamily of proteins that includes band 4.1 protein in erythrocytes, talin, the ezrin-radixin-moesin (ERM) family and also shows homologies to the family of protein tyrosine phosphatases. The ERM proteins have a common structural organization: a globular N-terminal domain followed by a long α-helical structure and a charged carboxy-terminal domain.

Schwannomin's role as a tumor suppressor is well established. However the pathway of signal transduction where schwannomin acts remains unclear. The lack of understanding about the function of the Nf2 protein is the foremost impediment in the development of treatments for Nf2. Therefore, we have focused on identifying proteins interacting with the Nf2 tumor suppressor schwannomin in an effort to elucidate schwannomin function. We have previously shown that schwannomin interacts with βII-spectrin demonstrating a role for schwannomin in cytoskeletal organization (Scoles et al., 1998). More recently, we identified the HGF regulated receptor substrate HRS as a schwannomin binding protein. Our research demonstrated that HRS and schwannomin co-localize to early endosomes and may have a role in regulating protein trafficking (Scoles et al., 2000).

We have also demonstrated that schwannomin interacts with the eukaryotic initiation factor 3 (eIF3) p110 subunit eight and now investigate the role of schwannomin in eIF3 function. The role of schwannomin in eIF3 function remains unknown, but because eIF3 is an essential element in the initiation of protein translation, schwannomin may regulate protein translation through interaction with p110. eIF3 is required for the 40 S ribosomal subunit bound to the ternary complex (eIF2-GTP-methionine) to interact with the 5' end of the mRNA, and is also needed to maintain the 40 S ribosomal subunit in the dissociated state (Seal et al., 1989). Very little of the function of the p110 subunit of eIF3 is known. The p110 interacts specifically to eIF1, resulting in eIF1 recruitment to the 40 S ribosomal subunit (Fletcher et al., 1999). eIF1 is required for positioning the complex at the initiation codon. The *Saccharomyces cerevisiae* ortholog of p110 is Prt1p which when mutated in *S. cerevisiae* results in inhibition of initiation of protein translation (Evans et al., 1995). Depletion of yeast p110 results in marked reduction of cell proliferation (Vornlocher et al., 1999).

We have made significant advancements on our research with p110 and schwannomin. We have developed new p110 antibodies and used them to further validate that schwannomin and p110 interact. We have also shown co-localization of p110 and schwannomin by confocal microscopy supporting their interaction. In addition, we have verified the interaction of schwannomin and p110 by using the non-transcriptionally-based "ras-rescue" yeast two-hybrid system. We have established systems to detect a direct physical interaction between schwannomin and p110 *in vitro*. Using yeast two-hybrid methods, we have further narrowed the region of Nf2 interaction in p110, and we have shown the effects of several Nf2 mutations on the schwannomin-p110 interaction. Finally, we have developed tet-inducible cell lines for the regulated expression of schwannomin in MEF cells and are now poised to make similar lines that induce p110.

Body

"Ras Rescue" two-hybrid verification of p110-schwannomin interaction

We have used the non-transcriptionally based "ras rescue" yeast two-hybrid system (CytoTrap, Stratagene) to verify that p110 and schwannomin interact. This element of our research does not relate to any Aims of the proposal, but very nicely provides additional validation of schwannomin-p110 interaction using a relatively new system in our laboratory. The method allows the determination of protein-protein interactions in a CDC25 mutant yeast strain unable to grow at 37°C. The advantage of the technique is that it allows the testing of proteins in yeast without the requirement that expressed fusion proteins translocate to the nucleus to activate transcription. Because schwannomin localizes to the cytoplasmic side of the plasma membrane in Schwann cells and many cell types, the method is especially applicable for the study of schwannomin since interactions tested with schwannomin in this system are accomplished at a functionally relevant location. To test interactions using the method, one protein to be tested is expressed as a fusion to a myristylation signal that targets the fusion protein to the plasma membrane using the plasmid pMyr. The other protein is expressed as a fusion to the SOS protein using the plasmid pSOS. Interaction between the two fusion proteins positions SOS at the membrane reconstituting the ras pathway in CDC25 mutant yeast, allowing growth at 37°C. The positive interaction between schwannomin and p110 in the "ras rescue" system (Appendix, Figure 1) provides yet another validation of their interaction.

New p110 antibody (Aim 2, Task 1)

We have made a new p110 antibody for the study of p110 and schwannomin. This antibody was made by immunizing a mixture of two peptide antigens in two rabbits. Upon determining that a full immonologic response occurred in the animals, we affinity purified the p110 antibodies and validated their specificity by conducting preabsorption analysis (Appendix, Figure 2). Both antibodies strongly and specifically detected p110 proteins extracted from the human schwannoma cell line STS26T that was absent or highly reduced when peptide antigen was used to block the antibody's ability to recognize the endogenous protein on the western blot.

To further validate the specificity of the antibodies, we cloned three different full-length p110 constructs, two for expression in mammalian cells, and one for expression *in vitro*. We overexpressed myc-p110 in RT4 cells and showed that the anti-p110 antibody 2867.2 detected the exogenous p110 protein while little endogenous protein was detected (Appendix, Figure 3A). Subsequently we have shown that the p110 antibody can be used reliably to detect rat p110 from RT4 cells if sufficient protein is loaded on a western blot (not shown). The anti-p110 antibody 2867.2 strongly detected the human p110 in STS26T cells (Appendix, Figure 3A). In addition, we detected exogenous p110 in STS26T cells transfected with myc-p110 using anti-myc-HRP conjugate that is highly sensitive, and showed that the antibody is very useful for detection of myc-tagged proteins (Appendix, Figure 3B). We also used a pcDNA3.1his-p110 construct to express p110 as a fusion to the xpress tag (Invitrogen). We observed that p110 appears as a wider band when expressed in STS26T cells suggestive of a mobility shift of p110 when fused to the xpress epitope (Appendix, Figure 3A). We have also observed mobility shifts when schwannomin is expressed as a fusion to the xpress epitope (not shown). We also constructed a variety of pBluescript-p110 constructs for use in in vitro interactions subsequent to in vitro translations. We preliminarily present the expression of p110 from the longest of these constructs, expressing full-length p110, by *in vitro* translation from the T7 promotor of pBluescript demonstrating specific detection using a non-radioactive system, compared to a luciferase control (Appendix, Figure 3C).

New Nf2 antibody

We have made a new Nf2 antibody. This new affinity purified Nf2 antibody was made in a chicken and is specific in its detection and very useful for western blotting and is extremely sensitive (see Appendix, Figure 6). The epitope with which this peptide antibody was made is the same as for our well-characterized rabbit anti-schwannomin ab5990 used in our past published works (Scoles et al., 1998; Scoles et al., 2000).

Co-immunoprecipitation of schwannomin and p110

The acquisition of solid co-immunoprecipitation data using a cell type of high phenotypic significance is invaluable to any further research to be conducted in this research project. We have already presented strong data showing both the co-immunoprecipitation of schwannomin with a p110 antibody and of p110 with a schwannomin antibody in the original proposal. However, the p110 antibody used in these cursory studies was difficult to use, and this is why we spent significant time to establish new high-quality p110 antibodies. Now, having the new p110 2867 antibodies, we found it necessary to once again validate the co-immunoprecipitation of schwannomin and p110. We successfully co-immunoprecipitated endogenous schwannomin from STS26T cells with endogenous p110 using our p110 antibody 2867.2 validating the interaction (Appendix, Figure 4).

Schwannomin and p110 co-localize in STS26T cells (Aim 2, Task 3)

We have strongly co-localized endogenous schwannomin and endogenous p110 in STS26T cells using confocal microscopy. The immunofluorescent pattern was punctate with a predominance for colocalization at a perinuclear region (Appendix, Figure 5). The co-localization was accomplished by co-labeling with a monoclonal Nf2 antibody (Transduction Laboratories) and our anti-p110 antibody ab2867.1. Background staining was insignificant (not shown). We will continue to investigate the co-localization of p110 and schwannomin in STS26T cells with our chicken anti-schwannomin antibody and in other cell lines, including inducible lines as they are developed.

Development of inducible cell lines (Aim 3, Task 2)

Using a retroviral system that we have recently established in the laboratory, we have successfully constructed a variety of tet-off MEF cell lines that express schwannomin isoform I or schwannomin isoform II under regulation of the TRE element. The schwannomin expressed in these lines are entirely of the wild-type sequence with no epitope tags. Expression is high so that the need for the epitope tag is absent. The expression of exogenous schwannomin in these cell lines is nearly totally eliminated by the addition of doxycycline (Appendix, Figure 6). These cells will become particularly useful for the analysis of the effect of schwannomin isoforms in altering the rate of proliferation (Aim 3, Task 2).

We have attempted to produce a regulatable p110 tet-off MEF cell line as well but we ran into difficulties. We observed that the endogenous p110 protein in these cell lines obscured the detection of exogenous p110 upon induction. This hampered our ability to select highly expressing lines. Consequently we have constructed the myc-p110 fusion protein construct and established its expression in mammalian cells and we also obtained an extremely sensitive detection system for the myc epitope tag (See above and Appendix Figure 3A,B). We have cloned this fusion protein into the retrovirus producing vector and are now prepared to construct myc-p110 regulated tet-off MEF cells.

We recognize that MEF cells may not be the cell type of choice for these experiments. Therefore, we are in the process of developing a tet-off regulated system using our human STS26T schwannoma cell line. We have already established a mixed population of tet-regulatable STS26T cells that includes high regulated expression based on preliminary experiments using a luciferase control, and are now in the process of fishing out a clonal line that will allow strong, specific regulation. When completed, we will establish tet-regulated p110 and schwannomin inducible STS26T cell lines.

Narrowing the interacting domains in schwannomin and p110 (Aim 1, Task 1)

We have used the yeast two-hybrid system to further narrow the interaction between schwannomin and p110 to include residues 327-690 of p110 (Appendix, Figure 7). To bolster our findings by the yeast two-hybrid, and to validate the interactions are direct, we have cloned eleven fragments of p110 in pBluescript and will use these clones for *in vitro* expression of p110 (Appendix, Figure 8). We will use the products of *in vitro* translation in binding assays with bacterially purified schwannomin. We have conducted the *in vitro* assay with the longest of these pBluescript clones, the full-length p110, and showed that it indeed expresses the full-length p110 protein as detected by a non-radioactive method (Appendix, Figure 3C).

Effect of Nf2 mutation on p110 affinity by schwannomin (Aim 1, Task 2)

We used the Gal4-based yeast two-hybrid system to test the effects of eleven *Nf2* missense mutations on p110 interaction. *Nf2* mutations significantly altered schwannomin binding to p110. Schwannomin isoform 2 mutated at L46R had reduced or no binding to p110 which binds the schwannomin N-terminal half. L46 is within the region involved in N-terminal domain folding (Gutmann et al., 1999). Schwannomin binding to p110 was abolished by mutations K364I, L535P, and Q538P. The crystal structure of moesin showed that the surface residues where contact is made between the N- and C-terminal domains share 81% identity to schwannomin, suggesting a similar mechanism in schwannomin folding (Pearson et al., 2000). Because moesin residues V518 and H521, which lie on the interface where the moesin N- and C-terminal domains make contact (Pearson et al., 2000), correspond to schwannomin residues L535 and Q538, schwannomin mutations at these locations are likely to alter schwannomin folding and binding properties.

Key Research Accomplishments

Our most significant accomplishment without question is the validation of the interaction between p110 and schwannomin by co-immunoprecipitation of the endogenous proteins with our newest Nf2 and p110 antibodies. This is a very significant accomplishment validating the entire study. With this information, p110 does indeed appear to be a true schwannomin interactor. This interaction is further bolstered by the ras rescue two-hybrid validation, all supporting the p110-schwannomin interaction.

We consider the acquisition of our newest p110 and schwannomin antibodies as key accomplishments as well. These new tools will greatly simplify our ability to characterize the roles of p110 and schwannomin in cell growth suppression roles.

Lastly, we consider the bringing online in our laboratory of the retroviral method for infection of transgene constructs as a major accomplishment. The method is relatively quick and simple and we are obtaining excellent results using the retroviral system.

Reportable Outcomes

All of the data presented in the Appendix is reportable. We are not yet prepared to proceed to publication with this information as the story will be considerably strengthened with the addition of validation that the p110-schwannoin interaction is direct between the two proteins by *in vitro* interaction. Additionally, with the *in vitro* data and a functional element (Aim 3, to be done in the future), an excellent quality publication likely will result.

We have prepared the mutation data (Appendix, Figure 9) for publication and are ready to submit this information right away. This publication will compare the effects of Nf2 mutations to alter schwannomin interactions with βII-spectrin, p110, two isoforms of HRS, and schwannomin itself.

Conclusions

We strongly conclude that p110 and schwannomin are interacting proteins. We coimmunoprecipitated schwannomin and p110 from human STS26T schwannoma cells and we colocalized schwannomin and p110 to the same perinuclear region within STS26T cells.

We further conclude that Nf2 gene mutation results in mutant schwannomin that is unable to bind p110, but that not all Nf2 gene mutations alter p110 binding, and that some Nf2 gene mutations outside the known binding region for p110 significantly alter p110 interaction. These data lead us to hypothesize that schwannomin folding—even in isoform 2 thought to be an unfolded isoform—disrupted by C-terminal mutation, is important for the proper maintenance of p110 interaction with the schwannomin N-terminal half.

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Appendix

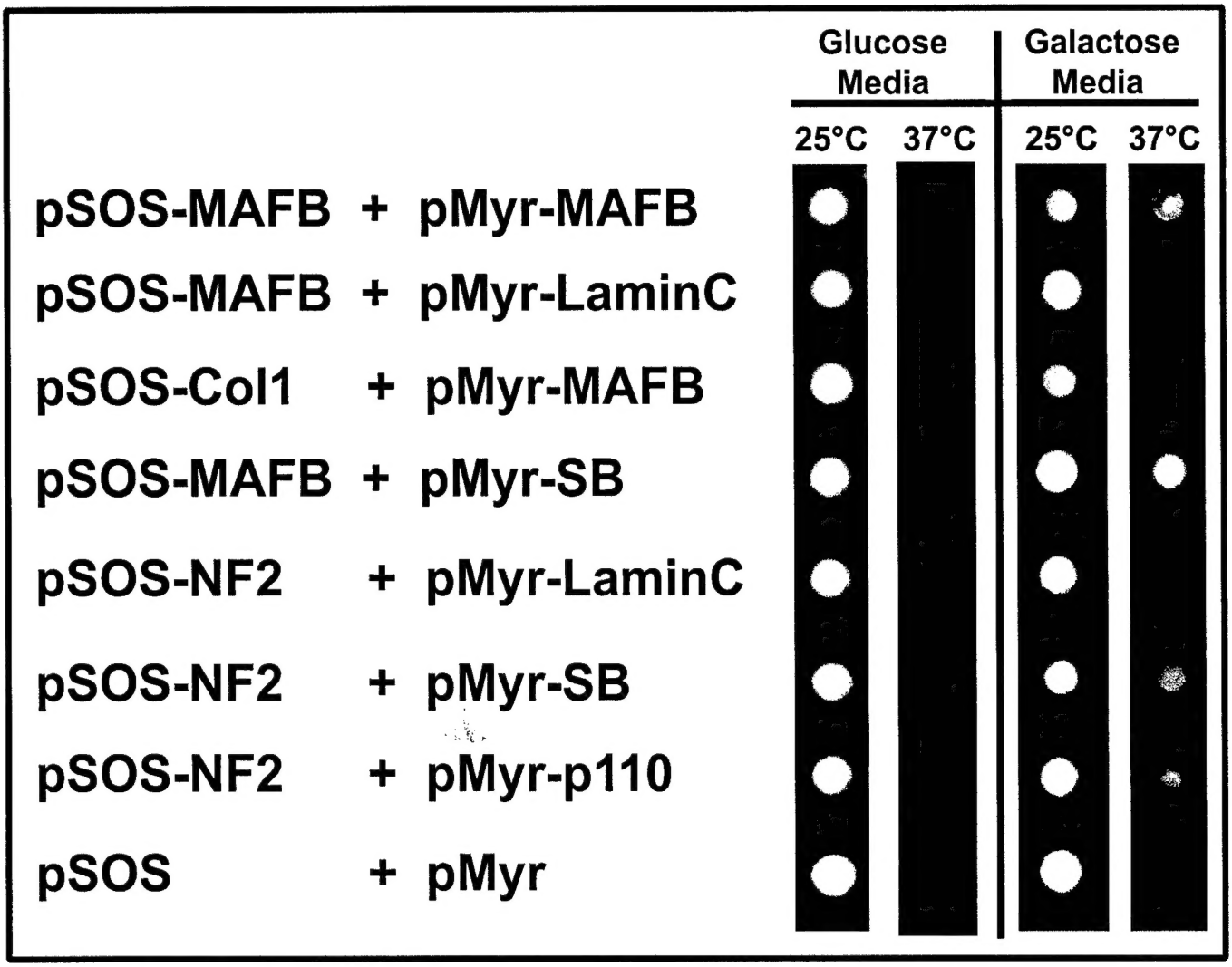

Figure 1

Validation of the p110-schwannomin interaction by using the "ras rescue" yeast two-hybrid method. This method allows the determination of protein-protein interactions in a CDC25 mutant yeast strain unable to grow at 37°C. We expressed p110 or a variety of controls as a fusion to a myristylation signal that targets the fusion protein to the plasma membrane using pMyr. We co-expressed schwannomin or a variety of controls as a fusion to the SOS protein using pSOS. Interaction between p110 and schwannomin positions SOS at the membrane reconstituting the ras pathway in CDC25 mutant yeast, allowing growth at 37°C. All co-transformants grow at 25°C on glucose or galactose (see figure). Because the promotor that drives fusion protein expression in pMyr is galactose inducible and glucose repressible, true interactors allow growth on galactose media at 37°C but not on glucose media at 37°C. MAFB is the maf-b homodimerizing oncoprotein. Human laminC and collagenase I were used as negative controls and none were positive for growth at 37°C. SB is the SOS binding protein used to verify that pSOS fusions have intact SOS. At 37°C, yeast transformed with pSOS-NF2 and pMyr-SB grew equally well as yeast transformed with pSOS-NF2 and pMyr-p110 validating schwannomin-p110 interaction in the system. We replicated the growth pattern eight times.

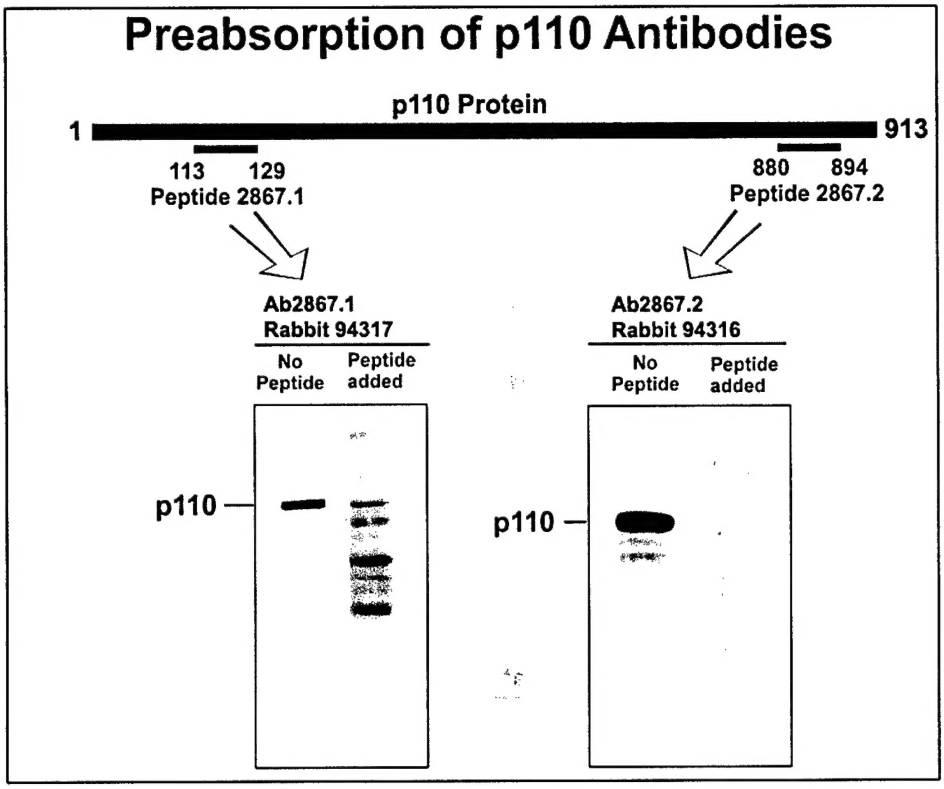

Figure 2

Characterization of specificity of new p110 antibodies. Two rabbits were immunized with a mixture of two different p110 peptides. We affinity purified the resultant antibodies and validated their ability to detect p110 in STS26T extract. Antibody 2867.1 from rabbit 94317 detected a single p110 band (left lane, left panel) of the same size as that detected by antibody 2867.2 from rabbit 94316 (left lane, right panel). Immunoreactivity of each of the antibodies is eliminated by preabsorption to its respective peptide antigen (right lanes of both panels). Ab2867.2 preformed the best of the two antibodies.

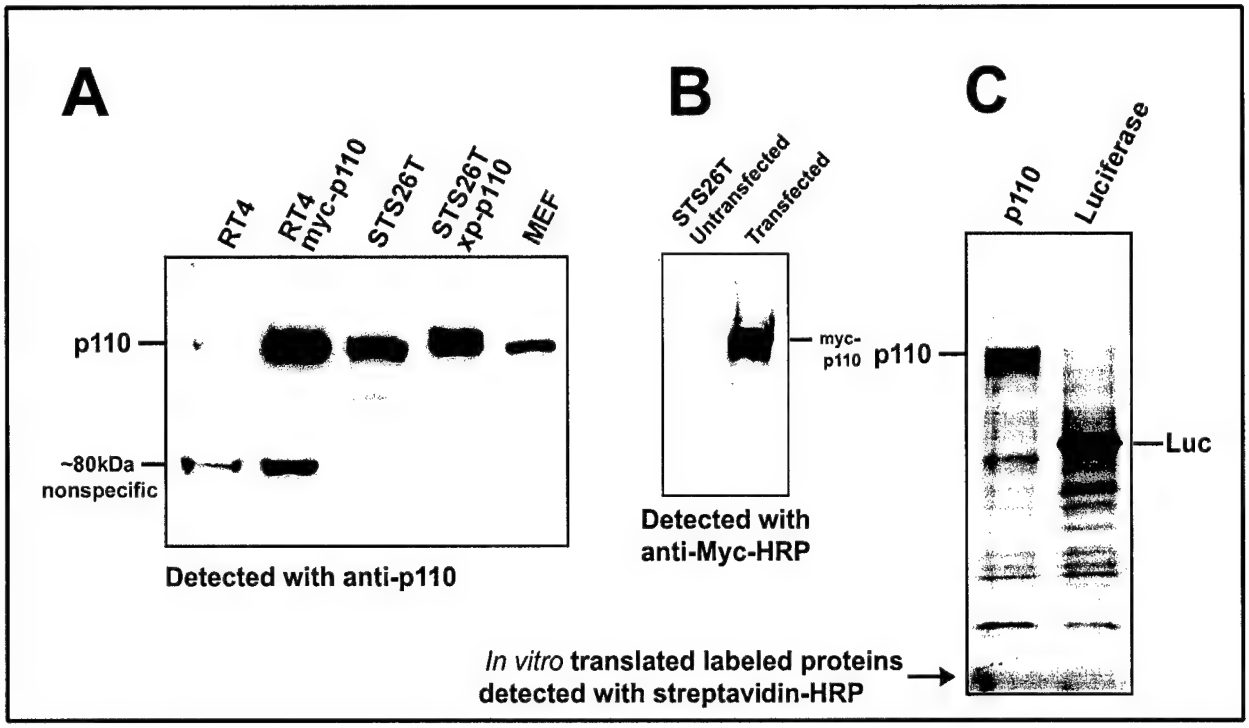

Figure 3

Anti-p110 ab2867.2 specifically detects endogenous and exogenous p110. We cloned the full-length p110 plasmid by screening a lambda phage library. The figure shows the results of three different full-length clones of p110: pCMV-myc-p110 to express a myc-p110 fusion protein, pcDNA3.1his-p110 to express xp-p110, a fusion to the xpress tag, and pBluescript-p110 used to *in vitro* translate p110. **A)** Anti-p110 very weakly detected endogenous p110 expressed in the rat schwannoma cells RT4 whereas a strong myc-p110 exogenous band was observed. Anti-p110 strongly detected human p110 in STS26T cells and mouse embryo fibroblasts (MEF). A larger band in xp-p110 transfected STS26T cells was observed compared to untransfected cells suggesting a mobility shift for this fusion protein. **B)** We used a very sensitive anti-myc-HRP conjugate to detect myc-p110 overexpressed in STS26T cells and found the antibody was highly specific detecting no band in untransfected cells. **C)** We translated and biotinylated p110 and luciferase *in vitro* and detected the proteins using streptavidin-HRP to demonstrate our pBluescript-p110 expresses a full-length protein.

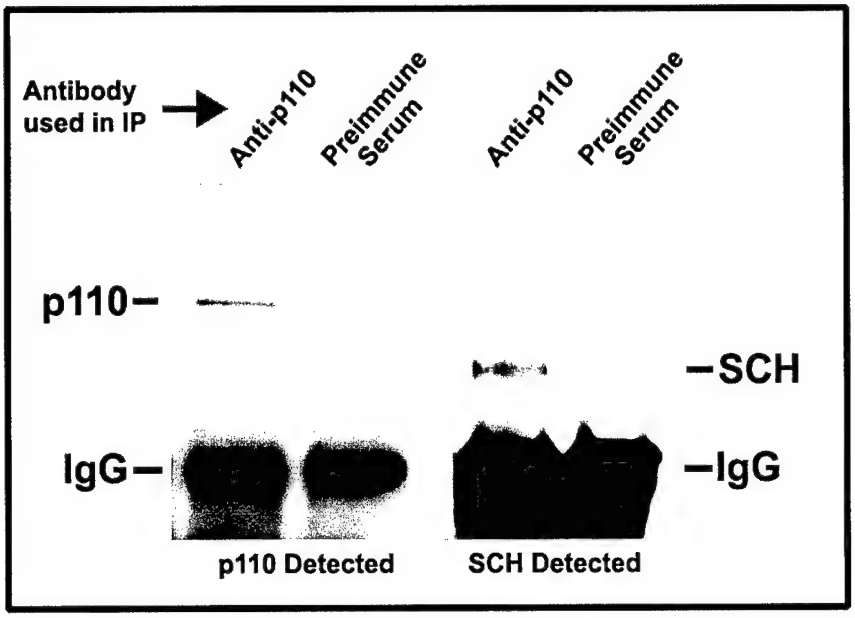

Figure 4

Anti-p110 ab2867.2 specifically immunoprecipitated endogenous schwannomin with endogenous p110. We extracted untransfected STS26T cells in CoIP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% nonidet-NP40, 0.5% deoxycholic acid), cleared non-specific binding proteins by incubating with agarose-rabbit IgG, and incubated with 1.5 μg ab2867.2, or an equal quantity of preimmune serum, overnight at 4°C. We captured immune complexes using protein-A/G resin and detected proteins on 4-15% gradient gels. The left blot was detected with ab2867.2 showing specific p110 immunoprecipitation. The right blot is the same preparation detected with our chicken anti-schwannomin showing specific detection of schwannomin. We conclude that schwannomin specifically co-immunoprecipitated with p110.

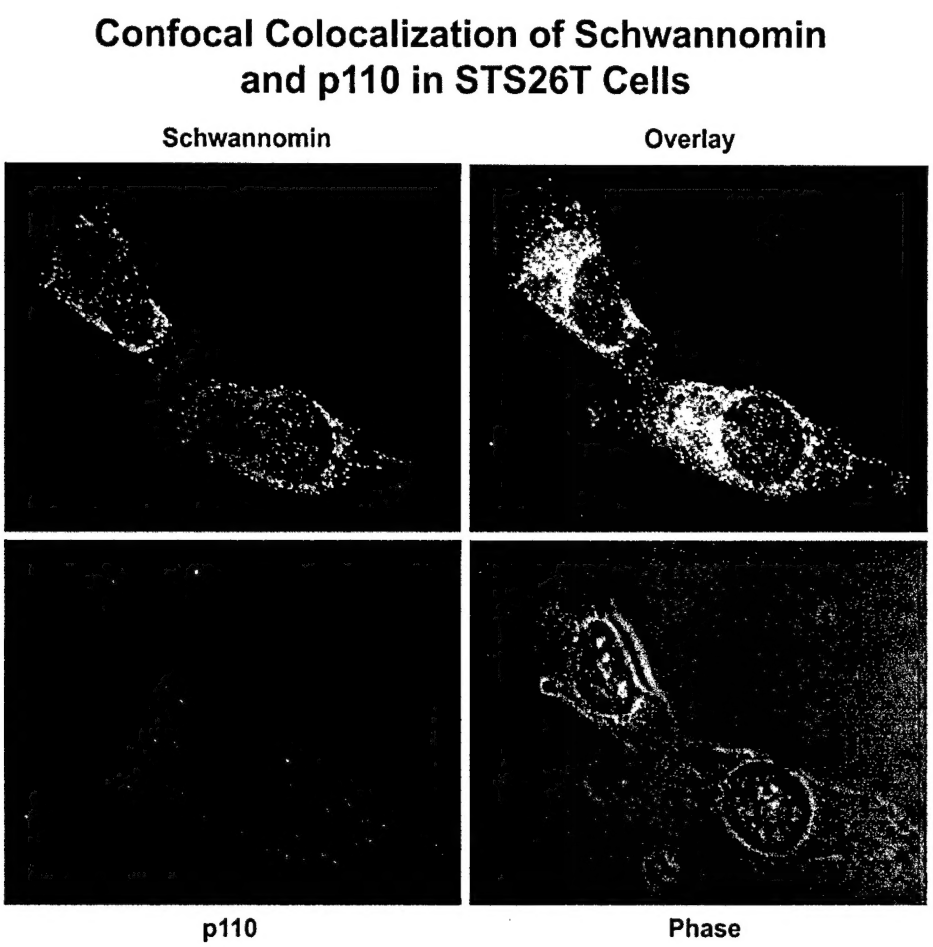

Figure 5

Confocal microscopy demonstrated strong co-localization of endogenous p110 and endogenous schwannomin in STS26T schwannoma cells. Schwannomin was detected with a monoclonal NF2 antibody and p110 was detected with ab2867.1. Co-localization of p110 and schwannomin was most pronounced at a perinuclear region. Confocal images were taken on a Zeiss LSM 310 confocal microscope.

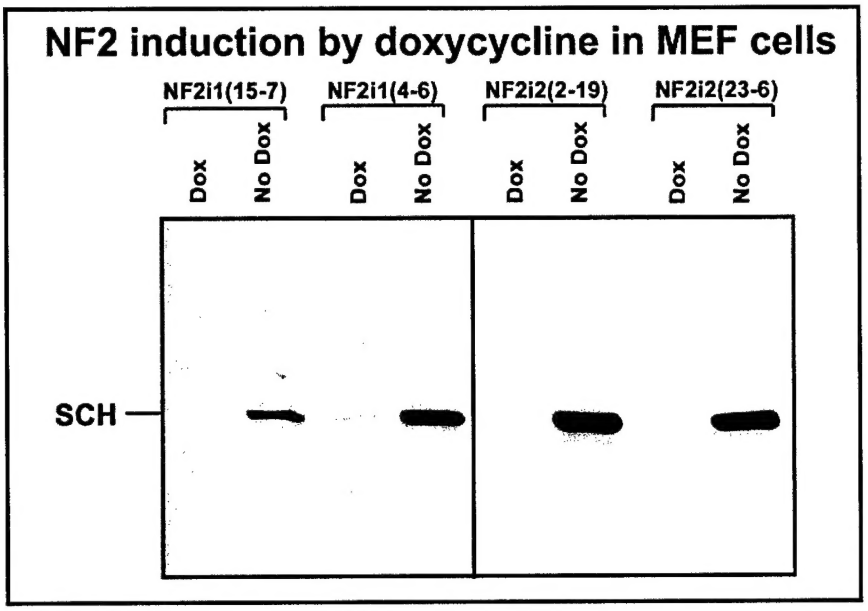

Figure 6

Establishment of NF2 tet-inducible mouse embryo fibroblasts (MEF) cell lines. We have established a system for the high-level tet-off inducible expression of schwannomin in MEF cells. We generated NF2 tet-inducible MEF cells by infecting generic tet-off MEF cells with a retrovirus carrying the TRE-NF2 isoform 1 or TRE-NF2 isoform 2 gene (human, untagged NF2 genes). After five rounds of viral infection, we put cells under antibiotic selection, and we screened the resultant colonies for specific schwannomin induction. This figure shows two lines of each isoform and demonstrates near total shut-down of transgene expression by treatment of 2 μg/ml doxycycline for 24 hrs. The blots were detected with chicken anti-schwannomin.

Yeast two-hybrid test of interactions

	pGBT9-NF2i1	pGBT9
pGAD10-p110(68-635)	+++	-
pGAD10-p110(68-300)	-	-
pGAD10-p110(230-465)	-	-
pGAD10-p110(327-690)	++	-
pGAD10	-	-

Figure 7

Narrowing of the NF2 binding region in p110 by yeast two-hybrid system. We used the Gal4-based yeast two-hybrid system to narrow the region where schwannomin binds p110 to p110 residues 327 to 635.

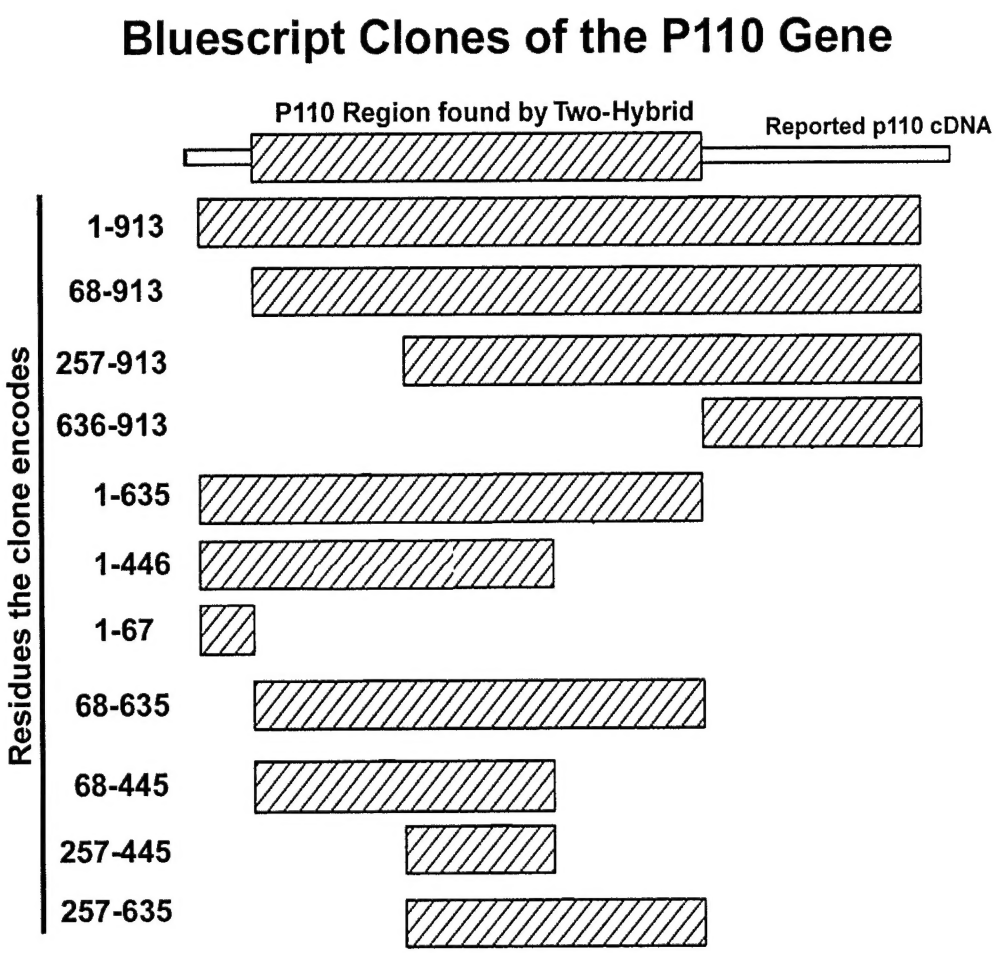

Figure 8

Regions cloned in pBluescript for *in vitro* expression of p110. We have made eleven clones of p110 in pBluescript with the intention of using these in *in vitro* translations. Please see Figure 3 for preliminary data on *in vitro* translation using the full length (aa1-913) pBluescript-p110 clone. We will translate p110 *in vitro* and test them for interaction with affinity purified, bacterially expressed schwannomin.

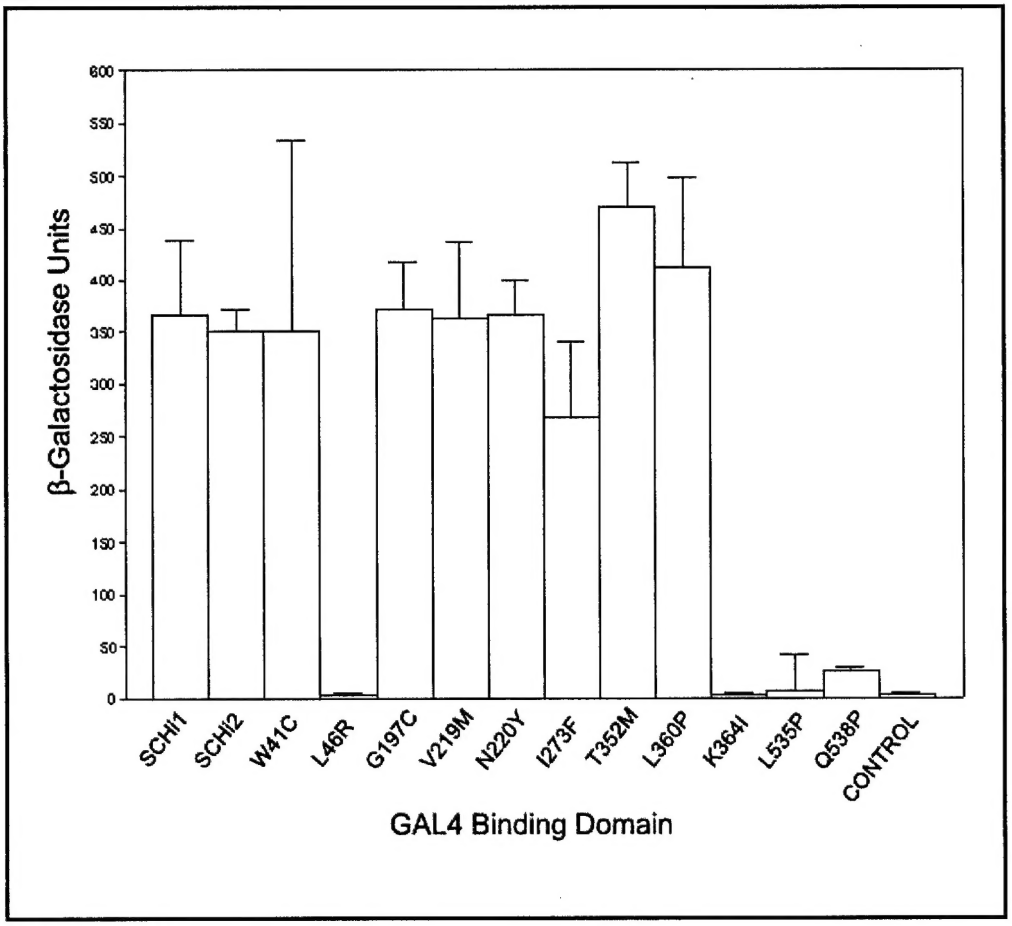

Figure 9

Effect of NF2 mutation on p110 affinity by schwannomin. We used the Gal4-based yeast two-hybrid system to test interaction between p110 and schwannomin isoform 1, isoform 2, or eleven schwannomin isoform 2 proteins with naturally occurring missense mutations. Although p110 binds within schwannomin residues 1-304, only one mutation within this region, L46R, abolished p110 binding. Five other mutations in this region had no effect on p110 binding while three mutations that alter the schwannomin C-terminal end of both isoforms resulted in no p110 interaction. Since p110 binding is altered by mutations that occur outside of its binding site in schwannomin, schwannomin conformation is likely important for p110 interaction.